Award Number: W81XWH-15-1-0491

TITLE: LIGHT-ing up prostate cancer for immunotherapy

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	2Sep2015 - 1Sep2016

4. TITLE AND SUBTITLE

LIGHT-ing up prostate cancer for immunotherapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0491

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNIVERSITY OF SOUTHERN
CALIFORNIA
UNIVERSITY GARDENS STE 203
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Recent discoveries have demonstrated that the expression of LIGHT molecules within the tumor milieu counteracts cancer immune-evasion mechanisms and instigates activation and migration of T-cells into the tumor; however the delivery of LIGHT to the tumor microenvironment has been a challenge. The overall goal of this project is to develop a targeted therapy that will deliver LIGHT to the tumor microenvironment by generating bispecific fusion proteins that are one-part LIGHT and one-part tumor-vasculature targeting antibodies (specifically targeting Delta-Like Ligand 4 (DLL4) and EphB4). In addition to LIGHT delivery, treatment with these antibodies alone has been shown to reduce tumor burden, which may enhance the effects of targeted LIGHT therapy. These bi-specific fusion proteins will be used, as proof-of-concept, to show that they lead to tumor eradication in both primary and advanced stage prostate cancer. In this annual report we summarize where we are in the production cycle and validation of these fusion proteins as well as provide a timeline for *in vivo* experiments.

15. SUBJECT TERMS
Regulatory T cells, prostate cancer, immunosuppression, tumor microenvironment, immunotherapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Prostate cancer is the second most common cause of cancer related deaths in men, with approximately 32,000 deaths expected annually in the United States and 258,000 deaths expected annually worldwide (1). While treatments for primary prostate cancer patients exist, many lead to devastating side effects such as impotence or incontinence and there are limited options for patients with advanced disease (2, 3). Thus, there is an obvious need for new therapies and immunotherapy has been proposed as a possible solution. The ultimate goal of cancer immunotherapy is to stimulate the patient's immune system to eradicate malignant tumors. However, the use of cancer vaccines alone has had limited curative success. A highly vascularized tumor microenvironment being advantageous for tumor growth, lack of T cell homing to solid tumors and tumor-mediated immune suppression if the T cells do infiltrate are critical barriers to achieving complete tumor eradication (4). Delta-like Ligand 4 (DLL4) and EphB4 are two proteins involved in angiogenesis that are upregulated in tumor vasculature and tumors. Their expression in the tumor microenvironment allows specific targeting by antibodies or antibody fragments. Anti-DLL4 antibodies target the tumor vasculature and have been demonstrated to induce non-functional angiogenesis and in turn reduce tumor burden (5). Anti-EphB4 antibodies target EphB4 expressed on tumor cells and likewise mediate tumor cell death (6). LIGHT is a costimulatory molecule that we have recently shown inhibits regulatory T cell (Treg) mediated immunosuppression and synergizes with prostate cancer vaccines when expressed in mouse prostate tumors when delivered intra-tumorally (7). Bispecific fusion proteins that engage both the tumor and T cells are promising candidates for cancer therapy because they guide T cells to the tumor site and enhance anti-tumor immunity. Expression of LIGHT in the tumor microenvironment through DLL4 or EphB4 antibody targeting is a novel approach to modify the primary tumor microenvironment and any distant metastases. This translational project proposes the development of two novel bi-specific fusion proteins – a single chain variable fragment of a DLL4 antibody (scFv-DLL4) fused to LIGHT and a single chain variable fragment of EphB4 (scFv-EphB4) fused to LIGHT – which will permit targeting of LIGHT to highly vascularized and advanced prostate tumors. These fusion proteins are expected to result in LIGHT expression in tumors converting an immunosuppressive tumor microenvironment to an immunostimulatory environment. Combining this with an immunotherapeutic prostate cancer vaccine will result in tumor antigen associated (TAA) specific T cells being able to migrate to tumors and receive costimulation by LIGHT, and avoid T cell inhibitory mechanisms such as Tregs.

KEYWORDS

- Prostate Cancer
- Immunotherapy
- Tumor Microenvironment
- LIGHT
- T regulatory cells
- Fusion proteins

ACCOMPLISHMENTS

Major goals of the project

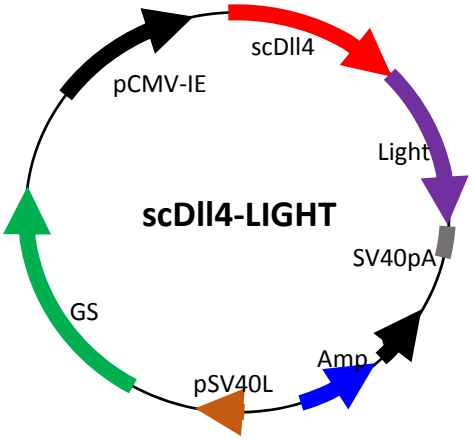
Specific Aim 1: To produce and validate scFv-DLL4-LIGHT protein and scFv-EphB4-LIGHT protein for in vivo studies.			
Aim 1.1: Produce a sufficient amount of scFv-DLL4-LIGHT protein and validate product for in vivo studies.	**Months**	**PI**	**Status**
Task 1: Establish a stable CHO cell line for scFv-DLL4-LIGHT expression	1-3	Gill	*Ongoing*
Task 2: Produce and purify a large amount of scFv-DLL4-LIGHT for animal experiments	4-6	Gill	*Ongoing*
Aim 1.2: Design and produce a sufficient amount of scFv-EphB4-LIGHT protein and validate product for in vivo studies.			
Task 1: Design and clone the scFv-EphB4-LIGHT plasmid construct	1-2	Kast	*Complete*
Task 2: Establish a stable CHO cell line for scFv-EphB4-LIGHT expression	4-6	Gill	*Ongoing*
Task 3: Produce and purify a large amount of scFv-EphB4-LIGHT for animal experiments	4-6	Gill	*Ongoing*
Milestone(s) Achieved: Production of two bi-specific fusion proteins that may target tumor vasculature and activate/recruit T cells.			*Ongoing*

Specific Aim 2: To determine the efficacy of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in TRAMP-C2 tumor bearing mice.			
Aim 2.1: Determine the dosage and duration of treatment for both scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in TRAMP-C2 challenged mice via tumor growth and survival.			
Task 1: Obtain IACUC/ACURO approval for planned mouse experiments	1-4	Kast	*Complete*
Task 2: Optimization of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT treatment schedule determined by survival and tumor burden.	6-12	Kast	*Pending*
Aim 2.2: Assess immunological mechanisms of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT treated TRAMP-C2 challenged mice and elect the more efficacious treatment.			
Task 3: Induction of tumor specific T cells against Tumor Associated Antigens.	13-17	Kast	*Pending*
Task 4: Isolation, frequency and phenotype of tumor-infiltrating cells (Th1, Th2, Tregs, NK, Th17, MDSC, macrophages).	17-20	Kast	*Pending*
Task 5: Compare the intra-tumoral cytokine and chemokine profile following treatment	17-20	Kast	*Pending*
Task 6: Evaluate Treg functionality post treatment.	17-20	Kast	*Pending*
Aim 2.3: Determine whether scFv-DLL4-LIGHT <u>or</u> scFv-EphB4-LIGHT treatment in combination with therapeutic prostate cancer vaccine can induce complete regression in TRAMP-C2 challenged mice.			
Task 7: Determine the most optimal vaccination scheme that will result in complete tumor control after combination treatment in TRAMP-C2 challenged mice.	20-24	Kast	*Pending*
Milestone(s) Achieved: IACUC/ACURO approval. Identification of the optimal dosage and duration, immunological mechanisms of scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in the TRAMP-C2 challenged setting. The more efficacious fusion protein (based on survival, tumor burden and immunological mechanisms) will be elected for further testing in combination treatments.			*Pending*

Specific Aim 3: Determine whether scFv-DLL4-LIGHT or scFv-EphB4-LIGHT treatment in combination with a therapeutic prostate cancer vaccine can induce complete regression of primary and advanced prostate tumors in the TRAMP model.			
Aim 3.1: Determine survival and tumor burden of primary prostate tumors in the TRAMP model after combination treatment.			
Task 1: Examine survival and tumor burden in combination treatment with MPSP TriVax and the elected bi-functional protein in primary prostate cancer (determined by Aim 2).	24-31	Kast	*Pending*
Aim 3.2: Determine survival and tumor burden of primary prostate tumors in the TRAMP model after combination treatment.			
Task 2: Examine survival and tumor burden in combination treatment with MPSP TriVax and the elected bi-functional protein in advanced prostate cancer (determined by Aim 2).	31-36	Kast	*Pending*
Task 3: Prepare and submit manuscript for publication.	35-36	Kast/ Gill	*Pending*
Milestone(s) Achieved: Efficacy of the elected bi-functional protein in combination with MPSP TriVax will be determined in primary and advanced disease. Manuscript submitted to high impact journal.			*Pending*

Accomplished goals during reporting period

Aim 1.1 – *Produce a sufficient amount of scFv-DLL4-LIGHT protein and validate product for in vivo studies:* Details of the expression vector are detailed below.



ATMRVPAQLLGLLLLWLPGARCDIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGV
PSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTGTVTFGQGTKVEIKRE**GGGGSGGGGSGGGGSGGGGS**EVQLVESGG
GLVQPGGSLRLSCAASGFTFTDNWISWVRQAPGKGLEWVGYISPNSGFTYYADSVKGRFTISADTSKNTAYLQMNSLRAE
DTAVYYCARDNFGGYFDYWGQGTLVTVSS**GGGGSGGGGSGGGGSGGGGS**RSHQANPAAHLTGANASLIGIGGPLLWETRLG
LAFLRGLTYHDGALVTMEPGYYYVYSKVQLSGVGCPQGLANGLPITHGLYKRTSRYPKELELLVSRRSPCGRANSSRVWW
DSSFLGGVVHLEAGEEVVVRVPGNRLVRPRDGTRSYFGAFMV

Color Legend:

Secretion signal

Dll4 Ab Light chain variable region

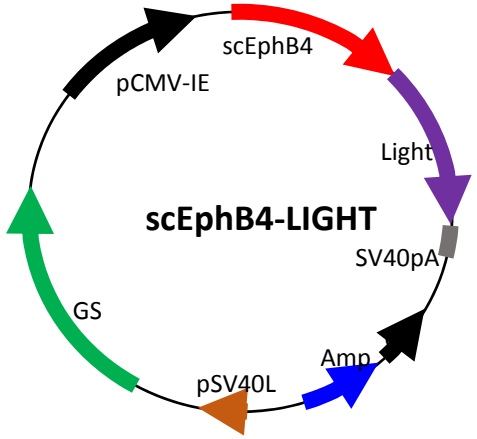
Dll4 Ab Heavy chain variable region

Mouse Light (R85-V239)

Expression vector was sequenced for accuracy and expressed transiently in CHO cells to confirm the expression. Purified protein was tested for binding to Dll4 to ensure that the fusion protein will bind to the target cells. Target cells include tumor vessels that highly express Dll4 while the normal tissue vessels do not express Dll4. Expression vector was then introduced in CHO-S suspension cells in chemically defined medium and a stable cell line was established. In order to ensure that the CHO-Dll4-LIGHT is stable, we are currently passaging the cell line and testing for the sustained expression of the fusion protein. Thirty passages are typically examined to ensure the stable expression. Once completed an expanded culture will provide bulk protein for purification and conduct of proposed aims.

Task 2: Produce and purify a large amount of scFv-EphB4-LIGHT for animal experiments.

Results: We have generated the expression vector to express a fusion protein of single chain EphB4 antibody and LIGHT as outlined below.



RVPAQLLGLLLLWLPGARCDIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSGTDFTLTISSLQP
EDFATYYCQESTTTPPTFGQGTKVEIKRGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFSISNYYLHWVRQAPGKGLEWVGGI
YLYGSSSEYADSVKGYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARGSGLRLGGLDYAMDYWGQGTLVTGGGGSGGGGSGGGGSGGGGSRS
HQANPAAHLTGANASLIGIGGPLLWETRLGLAFLRGLTYHDGALVTMEPGYYYVYSKVQLSGVGCPQGLANGLPITHGLYKRTSRYPKELELLVSRRSP
CGRANSSRVWWDSSFLGGVVHLEAGEEVVVRVPGNRLVRPRDGTRSYFGAFMV

Color Legend:

Secretion signal,

EphB4 Ab Light chain variable region

EphB4 Ab Heavy chain variable region

Mouse Light (R85-V239)

Expression vector was sequenced for accuracy and expressed transiently in CHO cells to confirm the expression. Purified protein was tested for binding to extracellular domain of EphB4-Alk Phos to ensure that the fusion protein will bind to the target cells. Target cells are primarily prostate cancer and some tumor vessel endothelial cells. Expression vector was then introduced in CHO-S suspension cells in chemically defined medium and a stable cell line was established. In order to ensure that the CHO-Dll4-LIGHT is stable, we are currently passaging the cell line and testing for the sustained expression of the fusion protein. Thirty passages are typically examined to ensure the stable expression. Once completed, the expanded culture will provide bulk protein for purification and conduct of proposed aims.

Task 3: Produce and purify a large amount of scFv-EphB4-LIGHT for experiments

Fusion protein purification is underway using size exclusion chromatography, and ion exchange chromatography, and hydroxyapatite. Protein mass will be measured by SDS using non-reducing conditions. Potency of the protein is measured by ELISA based soluble Dll4 and soluble EphB4 binding. These studies have been done in proteins expressed in transient expression system

Status: we are expanding the cell line

Aim 2.1 - *Determine the dosage and duration of treatment for both scFv-DLL4-LIGHT and scFv-EphB4-LIGHT in TRAMP-C2 challenged mice via tumor growth and survival.*

Task 1: Obtain IACUC/ACURO approval for planned mouse experiments Status: **Complete**

Results: We requested and received approval for the planned mouse experiments (20387-CR001).

USC Institutional Animal Care and Use Committee

Date: 6 Apr 2016

To: Martin Kast
MOLECULAR MICROBIOLOGY & IMMUNOLOGY
Diane DaSilva, PhD
Assistant Professor
MOLECULAR MICROBIOLOGY & IMMUNOLOGY

From: Institutional Animal Care And Use Committee
1540 Alcazar St, CHP 234
Los Angeles, CA 90033
(323) 442-1689

Title of Protocol: LIGHT-ing Up Prostate Cancer for Immunotherapy (20387-CR001)

The IACUC Committee reviewed and approved the above annual renewal on 4/6/2016.

Sincerely,

James Weiland, Ph.D.
Chair, IACUC

This is an auto-generated email. Please do not respond directly to this message using the "reply" address. A response sent in this manner cannot be answered. If you have further questions, please contact the IACUC office.

Opportunities for training and professional development:

Nothing to report

How were the results disseminated to communities of interest:

Nothing to report

What do we plan on doing to accomplish our goals during the next reporting period:

Produce and validate the fusion proteins (complete all tasks and milestones in Aim 1.1 and 1.2). After this is completed we will carry out Aim 2.1 and 2.2 to assess the optimal treatment concentrations of each fusion protein and describe, in detail, their immunotherapeutic functionality through the usage of the TRAMP-C2 challenge model (complete studies outlined Aim 2.1 and 2.2).

IMPACT

Impact on the development of the principal discipline of the project:

Nothing to report

Impact on other disciplines:

Nothing to report

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Nothing to report

CHANGES/PROBLEMS

Changes in approach and reasons for change:

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them:

We do not anticipate any problems with the planned studies including fusion protein production and studies. We are extensive experience in protein production, purification , thus we are capable of addressing any unforeseen issues.

Changes that had a significant impact on expenditures:

Nothing to report

Significant changes in use or care of vertebrate animals, biohazards, or other agents:

Nothing to report

PRODUCTS

Publications:

Nothing to report

Websites or other Internet Sites:

Nothing to report

Technologies or techniques:

Nothing to report

Inventions, patent applications, and/or licenses:

Nothing to report

Other products:

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals that have worked on the project:

Name	W. Martin Kast
Project role	Principal Investigator
eRA Commons	wmkast
Nearest person month worked	1
Contribution	Oversaw and provided guidance in projects progression.
Funding Support	NA

Name	Parkash Gill
Project role	Co-I
eRA Commons	parkashg
Nearest person month worked	1
Contribution	Overseeing generation of stable protein production cell lines and protein validation
Funding Support	NA

Name	Joseph G Skeate
Project role	Graduate student researcher
ORCID ID	0000-0002-1765-5949
Nearest person month worked	3
Contribution	Designed and cloned the scFv-EphB4-LIGHT plasmid construct, IACUC application
Funding Support	USC Keck School of Medicine/USC Graduate School PhD Fellowship

Name	Binyun Ma
Project role	Postdoc
eRA Commons	N/A
Nearest person month worked	1
Contribution	Responsible for generating stable protein production cell lines and protein validation
Funding Support	NA

Changes in active other support of the PIs or senior/key personnel since start of grant award:

Nothing to report

Other organizations that are involved:

Nothing to report

SPECIAL REPORTING REQUIREMENTS

Nothing to report

APPENDICES

References:

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer Journal international du cancer. 2010;127(12):2893-917. Epub 2011/02/26. doi: 10.1002/ijc.25516. PubMed PMID: 21351269.

2. Sivarajan G, Prabhu V, Taksler GB, Laze J, Lepor HL. Ten-year Outcomes of Sexual Function After Radical Prostatectomy: Results of a Prospective Longitudinal Study. European urology. 2013. Epub 2013/09/07. doi: 10.1016/j.eururo.2013.08.019. PubMed PMID: 24007711.

3. Ratcliff CG, Cohen L, Pettaway CA, Parker PA. Treatment regret and quality of life following radical prostatectomy. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2013. Epub 2013/08/03. doi: 10.1007/s00520-013-1906-4. PubMed PMID: 23907238.

4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74. Epub 2011/03/08. doi: S0092-8674(11)00127-910.1016/j.cell.2011.02.013. PubMed PMID: 21376230.

5. Djokovic D, Trindade A, Gigante J, Badenes M, Silva L, Liu R, Li X, Gong M, Krasnoperov V, Gill PS, Duarte A. Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis. BMC cancer. 2010;10:641. Epub 2010/11/26. doi: 10.1186/1471-2407-10-641. PubMed PMID: 21092311; PMCID: 3001720.

6. Krasnoperov V, Kumar SR, Ley E, Li X, Scehnet J, Liu R, Zozulya S, Gill PS. Novel EphB4 monoclonal antibodies modulate angiogenesis and inhibit tumor growth. The American journal of pathology. 2010;176(4):2029-38. Epub 2010/02/06. doi: 10.2353/ajpath.2010.090755. PubMed PMID: 20133814; PMCID: 2843490.

7. Yan L, Da Silva DM, Verma B, Gray A, Brand HE, Skeate JG, Porras TB, Kanodia S, Kast WM. Forced LIGHT expression in prostate tumors overcomes Treg mediated immunosuppression and synergizes with a prostate tumor therapeutic vaccine by recruiting effector T lymphocytes. Prostate. 2015;75(3):280-91. doi: 10.1002/pros.22914. PubMed PMID: 25399517; PMCID: PMC4306455.